(12) # EUROPEAN PATENT APPLICATION

(71) Applicant: TORAY INDUSTRIES, INC.
2, Nihonbashi-Muromachi 2-chome Chuo-ku
Tokyo 103(JP)

(72) Inventor: Tanaka, Toshiaki
31-22, Tsunishi 1-chome
Kamakura-shi Kanagawa(JP)

(72) Inventor: Kawano, Genji
3-13, Tsunishi 2-chome
Kamakura-shi Kanagawa(JP)

(72) Inventor: Sawada, Ritsuko
2-2-32, Katase
Fujisawa-shi Kanagawa(JP)

(74) Representative: Kador & Partner
Corneliusstrasse 15
D-8000 München 5(DE)

(54) Interferon conjugate and production thereof using recombinant gene.

(57) An interferon conjugates comprising in a single mole-
cule a region exhibiting biological activities of interferon-β
and a region exhibiting biological activities of interferon-γ;
deoxyribonucleotide sequences coding for the interferon
conjugate; recombinant DNAs comprising the deoxyribo-
nucleotide sequence and a deoxyribonycleotide sequence
coding for a control region for expression of the interferon
conjugate wherein the latter sequence is upstream from the
former sequence; transformant organisms transformed with
the recombinant DNA; and a process for production of the
above-mentioned interferon conjugate comprising the steps
of culturing the transformant organism to produce the
interferon conjugate, and recovering the interferon conju-
gate.

EP 0 237 019 A2

0237019

INTERFERON CONJUGATE AND PRODUCTION THEREOF
USING RECOMBINANT GENE

BACKGROUND OF THE INVENTION

1.   Field of the Invention

The present invention relates to interferon conjugates comprising interferon-β and interferon-γ, a

5   process for production of these interferon conjugates, and a gene system including a nucleotide sequence coding for these interferon conjugate, a recombinant DNA comprising the nucleotide sequence, and microorganisms transformed with the recombinant DNA.

10   2.   Description of the Related Art

Interferons are proteins exhibiting various biological activities, such as antiviral activity, and therefore, are promising for clinical application.

Interferons are classified into three types,

15   i.e., interferon-α, -β, and -γ, depending on their inducers, producer cells or antigenecities thereof, and are different in structure gene coding therefor, properties as protein, and in biological activities (Interferon no Kagaku (Science of Interferon), edited by

20   Shigeyasu Kobayashi, Kodan Sha).

Interferon-β (IFN-β) is a glycoprotein usually produced by fibroblast induced with virus or double-stranded RNA, is stable to treatment at pH 2, and unstable to treatment at 56°C.   Gene coding for

25   interferon-β has been already isolated (Taniguchi et al., Proc. Jpn. Acad., 55, Ser. B, 464-468, 1979).   The nucleotide sequence of the gene and a corresponding amino acid sequence have been determined, and moreover, a process for the production of interferon-β using cDNA

30   thus obtained and E. coli as a host has been developed (Taniguchi et al., Proc. Natl. Acad. Sci. USA, 77, 5230-5233, 1980; Goeddel et al., Nucleic Acids Res., 8, 4057-4074, 1980; and Derynck et al., Nature, 287,

193-197, 1980).

Interferon-γ (IFN-γ) is a glycopr tein usually produc d by T lymphocyte induc d with a mitogen, and is unstable to treatment at pH 2. A g ne coding for interferon-γ has been also isolated, the nucleotide sequence thereof has been determined, and a process for the production of interferon-γ using E. coli as a host has been established (Devos et al., Nucleic Acids Res. 10, 2487-2501, 1982; and Gray et al., Nature, 295, 503-508, 1982). Moreover, an amino acid sequence of native interferon-γ has been reported (Rinderknecht et al., J. Biol. Chem., 259, 6790-6797, 1984).

Among the interferons-α, -β, and -γ, interferons-α and -β have been known as interferon I, and it has been suggested that these interferons have a high structural analogy showing a 29% amino acid sequence homology (Taniguchi et al., Gene, 10, 11-15, 1980), and it is believed that they recognize same receptor. Therefore, the coexistence of interferons-α and -β provides their activities only in an additive manner. On the contrary, interferon-γ, known as interferon II, has a low amino acid homology with interferon I. It is believed that interferons I and II recognize different receptors (Branca et al., Nature, 294, 768-770, 1981). Therefore, interferons I and II exhibit different spectra of the anti-cell proliferation effect (Interferon no Kagaku (Science of Interferon), edited by Shigeyasu Kobayashi, Kodan Sha, Japan, 22-68, 1985), and exhibit their activities in a synergistic manner (Czarniecki et al., J. Virol., 49, 490-496, 1984; Fleishmann Jr. et al., J. IFN. Res., 4, 265-274, 1984; and Japanese Unexamined Patent Publication 59-98019).

Although a mixture of interferons-β and -γ will show the above-mentioned synergism in vitro, it is doubtful whether such synergism is established in vivo because there is some possibility that both interferon-β and -γ would not be simultaneously present at a target

site due to their different _in vivo_ pharmacodynamics.

To resolve the above-m ntioned disadvantage provided by the problems of _in vivo_ pharmacodynamics, it would be ffective to link interferons-β and -γ to make

5 a single polypeptide which could exhibit _in vivo_ the same synergism as shown _in vitro_ by a mixture of interferons-β and -γ. This means that, since a linked polypeptide molecule exhibits the same synergistically enhanced actions as shown by the parent two interferon

10 molecules, i.e., -β and -γ, the activity per a molecule is enhanced in comparison to the activity per molecule of the parent interferons, making it possible to obtain a new type of interferon with a higher activity.

Moreover, by causing a single polypeptide to

15 exhibit the different kinds of actions originally exhibited separately by interferons-β and -γ, it would be possible to obtain a new type of interferon having a broader spectrum of actions.

Some examples are already known wherein two

20 polypeptides having different actions are linked to make a single polypeptide having two actions (Yourno et al., Nature, 228, 820-824, 1970; Neuberger et al., Nature, 312, 604-608, 1984; and Bulow et al., Biotechnology, 3, 821-823, 1985). Moreover, insulin molecules were linked

25 to make a stable polypeptide (Shen and Shi-Hsiang, Proc. Natl. Acad. Sci. USA, 81, 4627-4631, 1984). As another example, there was disclosed a linkage of interferon-γ and interleukin-2 to make a single polypeptide which exhibits both actions of interferon-γ and interleukin-2

30 (Japanese Unexamined Patent Publication No. 60-241890).

However, so far there has been no attempt to link interferon-β with interferon-γ to prepare a single polypeptide having at the same time both a broader spectrum of actions and higher activities.

35 SUMMARY OF THE INVENTION

Therefore, the present invention relates to interferon conjugates comprising interferon-β and

interf r n-γ, which hav a br ader spectrum f biological
actions, such as antiviral action, anti-cell pr lif-
ration action, and th like, that were originally
carried s parat ly by par nt p lypeptides, i.e.,
5 interferon-β and interferon-γ, and which exhibits in
vivo synergistically enhanced activities as shown in
vivo by a mixture of interferons-β and -γ.

More specifically, the present invention provides
an interferon conjugate comprising in a single molecule
10 a region exhibiting biological activities of interferon-β
and a region exhibiting biological activities of
interferon-γ.

The present invention also provides a deoxyribon-
ucleotide sequence coding for this interferon conjugate.
15 Moreover, the present invention provides a
recombinant DNA comprising the above deoxyribonucleotide
sequence and a deoxyribonucleotide sequence coding for
a control region for expression of the interferon
conjugate, wherein the latter sequence is present
20 upstream from the former sequence.

Moreover, the present invention provides a trans-
formant organism transformed with the recombinant DNA.

Moreover, the present invention provides a process
for production of the above-mentioned interferon
25 conjugate comprising the steps of culturing the trans-
formant organism to produce the interferon conjugate,
and recovering the interferon conjugate.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 represents an embodiment of an amino acid
30 sequence of human mature interferon-β;

Fig. 2 represents an embodiment of an amino acid
sequence of human mature interferon-γ;

Fig. 3 represents a structure of plasmid pKM6 for
expression of human interferon-β;

35 Fig. 4 represents a structure of plasmid p6huγ-Nl
for expression of human interferon-γ;

Fig. 5 represents a structure of plasmid pKM6-Cxho

into which an XhoI site has been introduced at th
3'-terminus of the human interf ron -β structural gen;
Fig. 6 repres nts a structure of plasmid p6huγN1-
CKpn into which a KpnI sit has been introduc d at the
3'-terminus of the human interferon-γ structural gene;
Fig. 7 represents a structure of plasmid
p6huγN1ΔBS-NHin into which a HinPI site has been
introduced to take out a human interferon-γ structural
gene;

Fig. 8 schematically represents a process for
construction of a plasmid for expression of an inter-
feron-γ·β conjugate;

Fig. 9 schematically represents a process for
construction of a plasmid for expression of an
interferon-β·γ conjugate;

Fig. 10 schematically represents a process for
construction of a plasmid for expression of an
interferon-γcβ conjugate;

Fig. 11 represents a nucleotide sequence coding for
an amino acid sequence of a spacer peptide;

Fig. 12 schematically represents a process for
construction of a plasmid for expression of an
interferon-βcγ conjugate;

Fig. 13 schematically represents a process for
construction of a plasmid for expression of an
interferon-γ·β conjugate in animal cells; and

Fig. 14 schematically represents a process for
construction of a plasmid for expression of an
interferon-γcβ conjugate in animal cells.

In Figures 3 to 10 and 12, the numbers 1, 2, and 3
represent a human interferon-β structural gene, a human
interferon-γ structural gene, and a non-coding region of
human interferon-γ cDNA, respectively.

DESCRIPTION OF THE PREFERRED EMBODIMENT

The interferon conjugate of the present invention
comprises a region exhibiting biological activities
of interferon-β and a region exhibiting biological

activities f interfer n-γ linked directly r via a
spac r. According to the present invention, the terms
"interfer n-β" and "interfer n-γ" include any proteins
having activities specific to interf ron-β and

5 interferon-γ, respectively. For example, for
interferon-γ, a modified interferon-γ wherein its
polypeptide has been extended by three amino acid
residues at N-terminal (Gray et al., Nature, 295,
503-508, 1982), and a modified interferon-γ wherein a

10 part of the C-terminal of the polypeptide thereof has
been deleted (Rose et al., Biochem. J., 215, 273, 1983),
are known. These modified interferon-γ fall under the
scope of the present invention provided that they
maintain interferon activities. Moreover, modified

15 interferon-γ wherein some amino acid residues have been
replaced by other amino acid residues are disclosed
(Japanese Unexamined Patent Publications No. 59-93093
and No. 59-167596). These modified interferon-γ also
fall under the scope of the present interferon-γ provided

20 that they maintain interferon activities. Interferon-β
also can be modified by the addition, deletion or
replacement of one or more amino acid residues as
described for interferon-γ. Preferably, interferon-β
comprises a polypeptide having the amino acid sequence

25 shown in Fig. 1, and interferon-γ comprises a polypeptide
having the amino acid sequence shown in Fig. 2.

Juxtaposition order of interferons-β and -γ in the
present interferon conjugate is not limited. That is,
the interferon conjugate can contain as its N-terminal

30 half a polypeptide of interferon-β and as its C-terminal
half a polypeptide of interferon-γ, and vice versa.

In junction region in an interferon conjugate, a
polypeptide of interferon-β and that of interferon-γ can
be linked directly, or indirectly via a spacer, such as

35 a peptide. The spacer peptide is preferably a peptide
comprising many hydrophilic amino acids, as shown in an
example for an enzyme wherein subunits of β-galactosidase

are linked via a spacer peptide (Kushinke et al, EMBO
J., _4_, 1067-1073, 1985). Moreover, a polypeptide which
links domains of a naturally occurring protein can be
used as a spacer peptide of the present invention. A
5 spacer peptide usually consists of up to 50 amino acids.
A preferred spacer peptide is, for example, a peptide
known as a switch-peptide of immunoglobulin molecule,
and also a peptide having the amino acid sequence:
Thr-Gln-Leu-Gly-Gln-Pro-Lys-Ala-Ala-Lys-Ser-Val-Thr.

10   In the present invention, a structure comprising a
polypeptide of interferon-β and a polypeptide of
interferon-γ is designated as "interferon conjugate".
More specifically, a structure comprising as its
N-terminal half a polypeptide of interferon-β and as
15 its C-terminal half a polypeptide of interferon-γ is
designated as "interferon-β·γ conjugate" (IFN-β·γ);
and a structure comprising as its N-terminal half a
polypeptide of interferon-γ, and at its C-terminal
half, a polypeptide of interferon-β, is designated as
20 "interferon-γ·β conjugate" (IFN-γ·β). On the other
hand, a structure comprising as its N-terminal half a
polypeptide of interferon-β and as its C-terminal half a
polypeptide of interferon-γ wherein both polypeptides
are linked via a spacer is designated as an "interferon-
25 βcγ conjugate" (IFN-βcγ); and a structure comprising as
its N-terminal half a polypeptide of interferon-γ and as
its C-terminal half a polypeptide of interferon-β
wherein both polypeptides are linked via a spacer is
designated as an "interferon-γcβ conjugate" (IFN-γcβ).
30   As processes of the production of an interferon
conjugate, there are a chemical synthesis wherein amino
acids are coupled in a predetermined order, and genetic
engineering process wherein a DNA coding for a target
polypeptide is designed and constructed and the target
35 polypeptide is expressed in appropriate transformed
cells. In the present invention, preferably the genetic
engineering is used because it enables an easy production

of a target polypeptide, though the means f obtaining a
target polypeptide is not limited to genetic engineering.

In the use of gen tic engineering for th production
of an interferon conjugate, a DNA fragment comprising
5 a nucleotide sequence coding for a polypeptide of
interferon-β and a nucleotide sequence coding for a
polypeptide of interferon-β, wherein these nucleotide
sequences are directly linked or indirectly linked via a
nucleotide sequence coding for a spacer peptide, are
10 prepared, and this DNA fragment is linked to appropriate
control regions for expression of the target polypeptide,
and the coding sequence is expressed in appropriate host
cells.

Any nucleotide sequence coding for an interferon
15 conjugate can be used. That is, if more than one codon
is present for an amino acid, any codon which codes for
the amino acid can be used. Preferably, the same
nucleotide sequences as those of the cDNAs of
interferons-β and -γ respectively are used (Taniguchi
20 et al., Gene, 10, 11-15, 1980; and Devos et al., Nucleic
Acids Res., 10, 2487-2501, 1982).

To obtain a DNA fragment having a nucleotide
sequence coding for an interferon conjugate, chemical
synthesis of the DNA, isolation and linking of genes
25 coding for interferons-β and -γ, and a combination of
such means can be used. The chemical synthesis of a DNA
fragment having a desired nucleotide sequence can be
achieved according to a conventional procedure (Edge et
al., Nature, 292, 756-762, 1981; and Tanaka et al.,
30 Nucleic Acids Res., 11, 1707-1723, 1983). DNA fragment
coding for interferon-β or -γ can be derived from
chromosomal DNA, or preferably, prepared as cDNA.
cDNA can be isolated according to a known procedure
(Taniguchi et al., Proc. Jpn. Acad., 55, Ser. B, 464,
35 1979; Goeddel et al., Nucleic Acids Res., 8, 4057-4074,
1980; Derynck et al., Nature, 287, 193-197, 1980; Devos
et al., Nucleic Acids Res. 10, 2487-2501, 1982; and Gray

et al., Nature, 295, 503-508, 1982). Alternatively, the desired DNA fragment can be obtained by screening a cDNA library prepared according to a known procedure (Okayama et al., Molecular and Cellular Biology, 3, 280, 1983)

5 and using a probe designed from a known nucleotide sequence described in the above-mentioned literature.

To obtain a DNA fragment coding for an interferon conjugate from cDNAs of interferons-β and -γ, the cDNAs are separately digested with an appropriate restriction

10 enzyme, and the resulting cDNA fragments are ligated immediately or after blunt-ending with a mung bean nuclease, DNA polymerase I Klenow fragment or T4 DNA polymerase. The cDNA fragments are preferably linked via a synthetic oligonucleotide corresponding to

15 nucleotides deleted by the restriction enzyme cleavage, to obtain a DNA fragment coding for an interferon conjugate comprising entire polypeptides of interferons-β and -γ. In such a case, according to one embodiment of the present invention, the synthetic oligonucleotide

20 consists of a nucleotide sequence coding for a spacer peptide flanked by nucleotide sequences corresponding to nucleotides deleted by the restriction enzyme cleavage, so as to obtain a DNA fragment coding for an entire interferon conjugate comprising polypeptides of

25 interferons-β and -γ linked via the spacer peptide. Alternatively, a restriction enzyme site is introduced in cDNAs of interferon-β and interferon-γ at their 5'-end or 3'-end of the structural gene using synthetic oligonucleotides, and the cDNAs are cleaved with an

30 appropriate restriction enzyme, and if necessary, after the cDNAs are blunt-ended, the cDNAs are ligated. In any case, one cDNA fragment should be linked in reading frame with another cDNA fragment.

To produce an interferon conjugate using the

35 above-mentioned DNA construct, animal or plant cells, yeast or E. coli are used as a host. To express an interferon conjugate, the DNA construct should have a

promoter for starting the transcription, and an SD
sequence and ATG codon for starting the translation
upstream therefrom. As a promoter, any nucleotide
sequence having a promoter activity can be used; for

5 example, lac promoter, trp promoter, recA promoter,
and the like are known. Preferably a strong promoter
such as trp promoter is used. The SD sequence is a
ribosome RNA binding site and, therefore, essential for
translation. In the present invention, any SD sequence

10 can be used. A control region comprising the above-
mentioned promoter and SD sequence thus constructed
is added to a DNA fragment coding for an interferon
conjugate via an ATG codon to express the interferon
conjugate. The addition of the ATG codon can be carried

15 out using synthetic oligonucleotides (Goeddel et al.,
Nature, 281, 544-548, 1979). Alternatively, for
interferon-β, the ATG codon can be exposed according to
a known procedure (Taniguchi et al., Proc. Natl. Acad.
Sci. USA, 77, 5230-5233, 1980).

20   To introduce the thus-obtained DNA into host cells,
a vector DNA is used. Vector DNAs used for an E. coli
host include a plasmid DNA such as pBR322 and pSC101,
and a phage DNA such as λ phage. Such a DNA vector is
linked with the DNA construct for expression of

25 an interferon conjugate to construct an expression DNA
vector, which is then used to transform host cells
according to a known procedure (Maniatis et al.,
"Molecular Cloning", Cold Spring Harbor Laboratory,
p250-255, 1982).

30   A transformant such as transformed E. coli is
then cultured in an appropriate medium to produce an
interferon conjugate. Culturing is preferably carried
out in a liquid medium. When trp promoter is used,
indoleacrylic acid is added to a culture medium during

35 culturing to induce the production of an interferon
conjugate. If a different promoter is used, an inducer
selected in accordance with the particular promoter is

pr ferably used, to enhance the production of an
interferon conjugate.

The interferon conjugate producer such as the
thus-cultured E. coli cells are then disrupted according
5 to a conventional cell disruption procedure
("Tanpakushitsu·Koso no Kisojikken" (Fundamental
Experiment of Protein and Enzyme), edited by Takekazu
Horio and Jinpei Yamashita, Nankodo, 1981, 3-7), for
example, by treatment with a enzyme, sonication,
10 grinding, or treatment under pressure, to obtain a crude
extract containing an interferon conjugate.

The above-mentioned cell disruption procedure can
be combined with treatment with guanidine hydrochloride
or urea to improve the extraction efficiency (Davis et
15 al., Gene, 21, 273-284, 1983).

The thus-obtained crude extract can be further
purified according to a known procedure
("Tanpakushitsu·Koso no Kisojikken" (Fundamental
Experiment of Protein and Enzyme), edited by Takekazu
20 Horio and Jinpei Yamashita, Nankodo, 1981, 18-382), for
example, by salting out, ultrafiltration, ion exchange,
gel filtration, affinity chromatography, electrophoresis,
or a combination thereof, to prepare a highly purified
interferon conjugate preparation.

25 To express an interferon conjugate in animal cells,
it is necessary that a nucleotide sequence coding for
the interferon conjugate be present under the control of
a promoter effective in the animal cells. Promoters
effective in animal cells include, for example, SV40
30 early promoter, SV40 late promoter, a promoter of a HB
virus gene, MMTV promoter, a promoter of a thymidine
kinase gene, a promoter of a heat shock protein, and a
promoter of an interferon gene. A nucleotide sequence
coding for an interferon conjugate is linked downstream
35 of one of the above-mentioned promoters in the same
manner as described for the expression in E. coli. A
single promoter or a combination of more than one

promoter can be used. Not , an enhancer sequence of
Harbey mouse sarcoma virus 5'LTR or an enhancer sequence
of SV40 can be linked upstream of the above-mentioned
promoter for a eukaryotic cell. Such enhancers are
5   believed to increase the transcription efficiency.
Preferably, a nucleotide sequence coding for signal
peptide for extracellular secretion is added just
upstream of the nucleotide sequence coding for an
interferon conjugate, extracellularly to secrete the
10   interferon conjugate, which can be then recovered in a
supernatant of culture broth.
    To prepare a large amount of the above-mentioned
DNA construct so that the DNA construct can be easily
introduced into animal cells, the DNA construct
15   preferably includes an E. coli replication origin and
a drug resistance gene. The replication origin is
preferably, but not limited to, that derived from a
colicin E1 plasmid, for example, pBR22, or a related
plasmid. The drug resistance gene is, for example, an
20   ampicillin resistant gene, tetracycline resistant gene,
or kanamycin resistant gene. Moreover, the DNA construct
can contain a replication origin responsible for
autonomous replication in host cells, for example, a
replication origin of SV40 virus or a replication origin
25   of polyoma virus. These various DNA fragments are
linked to construct an interferon conjugate expression
vector.
    The construction of a vector DNA can be carried out
according to a conventional procedure (T. Maniatis et
30   al., "Molecular Cloning", p86-96, 1982).
    Animal cells into which a vector DNA will be
introduced may be cells derived from humans, monkeys,
Chinese hamsters, mice, etc., although since a human
interferon conjugate is the target compound, human cells
35   are preferably used. Human cells wherein growth is not
inhibited by a peptide having glycoside chains produced
are used. The human cells are preferably cells derived

from human lung cancer, especially PC8 and PC12 cell
lines (M. Kinjo et al., Br. J. Cancer, 39, 15, 1979).

Vector DNA can be introduced into host cells
according to a known calcium phosphate method (F.L.
5 Graham et al., Virology, 54, 536, 1973).

Cells into which a plasmid for expression of
an interferon conjugate has been introduced can be
efficiently obtained by cotransformation of host cells
with the plasmid and G418 resistance gene expression
10 vector pSV2neo (P.J. Southern et al., J. Mol. Appl.
Genet., 1, 327, 1982) or pNE05' (M. Lusky et al., Cell,
36, 391, 1984), because the cotransformed cells can
survive in a medium containing G418, in which non-
cotransformed cells cannot survive.

15 The thus-obtained transformant is cultured in,
for example, a medium containing fetal calf serum,
extracellularly to produce an interferon conjugate,
which is then recovered in a pure form from a supernatant
of the cultured broth according to the same procedure as
20 described for recovery from the E. coli extract. The
thus-obtained interferon conjugate is a polypeptide
having glycoside chains.

Since the interferon conjugate prepared as described
above binds to an anti-human interferon-β antibody and
25 to an anti-human interferon-γ antibody, it has the
antigenecities of both interferons-β and -γ. Moreover,
a neutralization test thereof with anti-human
interferon-β antibody and anti-human interferon-γ
antibody showed that a single polypeptide exhibits both
30 interferon-β activity and interferon-γ activity.

As described above, the present invention provides
a new type of interferon conjugate comprising a region
exhibiting biological activities of interferon-β and a
region exhibiting biological activities of interferon-γ,
35 which is produced by recombinant cells containing DNA
coding for interferon-β and interferon-γ.

The interferon conjugate of the present invention

is a singl polypeptide carrying biol gical actions
which were originally carried separately on interferon-β
and interferon-γ. Theref r , the present interfer n
conjugate has a broader action spectra, such as an
5 antiviral spectrum and an anti-cell proliferation
spectrum broader than those of conventional interferon.
These properties make the present interferon conjugate
valuable as an antiviral pharmaceutical or an antitumor
pharmaceutical.
10   Moreover, since the present interferon conjugate
exhibits the synergism conventionally provided by a
mixture of interferon-β and interferon-γ, it provides
remarkably enhanced activity per molecule in comparison
to conventional interferons. Although such synergism
15 can be obtained in vitro by only mixing interferon-β
and -γ, since interferons-β and -γ, would show different
in vivo pharmacodynamics, the coexistence of both
interferons at a target site is not assured in vivo.
This means that the mixture of both interferons-β and -γ
20 would not exhibit the synergism. On the contrary, in
the present interferon conjugate, since a single molecule
provides the synergism, the problem of different pharma-
codynamics does not exist. This also makes the present
interferon conjugate valuable as an antiviral pharma-
25 ceutical and an antitumor pharmaceutical.
        Finally, at present, to obtain interferon-β
activity and interferon-γ activity, separate processes
for the production of interferons-β and -γ must be
carried out. On the contrary, according to the present
30 invention, one production process provides an interferon
conjugate carrying both interferon-β activity and
interferon-γ activity. This gives an economical
advantage to the present invention. The present
interferon conjugate can be used as such, or can be
35 easily cleaved into interferon-β and interferon-γ, which
then can be used separately or as a mixture. In all
cases the above-mentioned advantage still remains.

<u>Examples</u>

The present invention will now be further illustrat d by, but is by no means limited to, the following examples.

5   Common procedures for gene manipuration used in the following Reference example and Examples are those described is Maniatis et al., "Molecular Cloning", Cold Spring Harbor Laboratory, 1982.

Prior to illustrating Examples of the present
10 invention, the construction processes of a plasmid for the expression of human interferon-β and a plasmid for the expression of human interferon-γ are described as a Reference example. These plasmids are used as starting plasmids for the construction of the present plasmids.

15   <u>Reference example</u>

(1)   <u>Construction of plasmid pKM6 for expression of human interferon-β.</u>

A plasmid pTuIFNβ-5 for the expression of human interferon-β constructed according to a reported
20 procedure (Taniguchi, Seikagaku, <u>54</u>, 363-377, 1982) was digested with HindIII, and the digestion product was treated with a T4 DNA polymerase Klenow fragment to make blunt ends, linked to a BglII linker, digested with BglII, and self-circularized using a T4 DNA ligase to
25 obtain a plasmid pYO-10. The plasmid pYO-10 was digested with SalI and ClaI, and the digestion product was subjected to agarose gel electrophoresis to isolate a DNA fragment of about 830 bp. This DNA fragment was inserted into a ClaI-SalI region of the plasmid p6huγ-A2
30 described in Japanese Unexamined Patent Publication No. 61-19488 to construct a plasmid pKM6 shown in Fig. 3.

(2)   <u>Construction of plasmid p6huγ-N1 for expression of human interferon γ.</u>

Lympocyte derived from human tensils was
35 treated with phytohemagglutinin (PHA) and 12-o-tetra-decanoyl-phorbor-13-acetate (TPA) to induce the production of human interferon-γ (Vilvek et al.,

Infection and Immunity, 34, 131, 1983), and then mRNA
was prepared from the treated cells. The preparations
of mRNA and cDNA, and the cloning of cDNA in a plasmid
were carried out according to known procedures (Okayama
5   et al., Molecular and Cellular Biology, 3, 280, 1983).
The DNA library thus obtained was subjected to colony
hybridization using a synthetic oligonucleotide probe
having the sequence: 5'-AGGACAACCATTACT-3' corresponding
to a region near the 3'-terminal of a known human
10 interferon-γ structural gene (Goeddel et al., Nature,
295, 503-509, 1982) to obtain a plasmid pIFN-γ15
containing a cDNA coding for human interferon-γ. Next,
after the plasmid pIFN-γ15 was digested with NdeI, and
BamHI, a DNA fragment of about 0.9 kb was isolated by
15 agarose gel electrophoresis.

On the other hand, two oligonucleotides
5'-CGATGCAGGACCCA-3' and 5'-TATGGGTCCTGCAT-3' were
synthesized, and after phospholyration at the 5'-end
with a T4 polynucleotide kinase, the oligonucleotides
20 were mixed to make a concentration of about 8 pmol/µl
for each oligonucleotide. The mixture was then heated
at 65°C for 3 munutes, cooled rapidly, heated again at
65°C for 3 minutes, and then allowed to stand at a room
temperature to be cooled gradually and to anneal the
25 oligonucleotides.

7 pmoles of this oligonucleotide, 0.3 pmole of
NdeI-BamHI fragment from plasmid pIFNγ-15, and 0.1 pmole
of a DNA fragment of about 4200 bp prepared by digestion
of the plasmid pKM6 described in paragraph (1) with ClaI
30 and BamHI followed by isolation by agarose gel electro-
phoresis were mixed, and after ligation the ligation
mixture was used to transform E. coli MC1061 (Casadaban
et al., J. Mol. Biol., 138, 179-207, 1980). The trans-
formants selected for ampicillin resistance were sub-
35 jected to colony hybridization using an oligonucleotide
probe 5'-TATGGGTCCTGCAT-3', to obtain a plasmid p6hur-N1
for expression of human interferon-γ (Fig. 4).

Next, to link the interferon-β structural gene
and interferon-γ structural gene, restriction enzyme
sites are introduced to the 5'-end or 3'-end of each
structural gene.

5   (3)   <u>Construction of plasmid pKM6-Cxho</u>
The structure of the plasmid pKM6-Cxho is
shown in Fig. 5. The plasmid pKM6 constructed as
described in paragraph (1) was digested with BstEII
and BamHI, and an adapter oligonucleotide having the

10 following sequence:

              GTTACCTCCGAAACTCGAGCTGA

                 GAGGCTTTGAGCTCGACTCTAG

was linked to the cleaved plasmid to construct the
plasmid pKM6-cxho. The adapter was prepared according

15 to the same procedure as described in paragraph (2).
When the plasmid pKM6-Cxho is digested with XhoI and the
protruding nucleotides are eliminated, a codon AAC
coding for aspartic acid present at the C-terminal of
human interferon-β is exposed.

20   (4)   <u>Construction of plasmid p6huγNl-CKpn</u>
The structure of the plasmid p6huγNl-CKpn is
shown in Fig. 6. The plasmid p6huγ-Nl constructed as
described in paragraph (2) was digested with ClaI and
BamHI, and after agarose gel electrophoresis, a DNA
25 fragment of about 4200 bp and a DNA fragment of about
1050 bp were isolated. The DNA fragment of about
1050 bp was further digested with HinfI, and after
agarose gel electrophoresis a DNA fragment of about
400 bp was isolated. The ClaI-BamHI fragment of about
30 4200 bp prepared as described above, the ClaI-HinfI
fragment of about 400 bp, and an adapter of the following
sequence:

  (5')-AGTCAGATGCTGTTTCGCGGTCGACGTGCATCCCAGGTACCATGAGATCTG

       GTCTACGACAAAGCGCCAGCTGCACGTAGGGTCCATGGTACTCTAGACCTAG-(5')

35 were mixed and ligated, and the ligation mixture was
used to transform <u>E. coli</u> MC1061. The above-mentioned
adapter was prepared from six oligonucleotides according

t the same procedure as described in paragraph (2).
Th transformants s lected for ampicillin resistance
were subjected to colony hybridization using an
oligonucleotide probe 5'-GATCCAGATCTCATG-3' t btain
four positive clones from 118 clones. The positive
clones contained plasmid p6huγ-CKpn. When the thus-
prepared plasmid p6huγ-CKpn is digested with KpnI, and
protruding nucleotides are eliminated, a codon CAG
coding for glutamine present at the C-terminal of human
interferon-γ is exposed.

(5) Construction of plasmid p6huγN1ΔBS-NHin

The structure of plasmid p6huγN1ΔBS-NHin is
shown in Fig. 7. The plasmid p6huγ-N1 constructed as
described in paragraph (2) was digested with BstEII, the
resulting cohesive ends were changed to blunt ends using
a DNA polymerase I Klenow fragment, and the resulting DNA
fragment was linked to a SalI linker. After digestion
with SalI, the DNA fragment was self-circularized using
the T4 DNA ligase to obtain p6huγN1-ΔBS. The plasmid
p6huγN1-ΔBS was digested with NdeI and SalI, and after
agarose gel electrophoresis, a DNA fragment of about
3700 bp was isolated.

On the other hand, the plasmid pKM6 constructed
as described in paragraph (2) was digested with EcoRI
and SalI, and after agarose gel electrophoresis, a DNA
fragment of about 3700 bp was isolated.

Two DNA fragments prepared as described above
were linked with an adapter oligonucleotide of the
following sequence:

AATTGCGCAGGACCCA

CGCGTCCTGGGTAT

to obtain plasmid p6huγN1ΔBS-NHin. When the plasmid
p6huγN1ΔBS-NHin is digested with HinPI, and protruding
nucleotides are eliminated, a codon CAG coding for
glutamine present at the N-terminal of human interferon-γ
is exposed.

Example 1. Construction of plasmid ptrp6huIFN-γβ

for expression of interferon-γ·β
conjugate

A process for the construction of a plasmid
ptrp6huIFN-γβ is shown in Fig. 8, wherein 30 μg of
5 plasmid pKM6 was digested with ClaI, and the digestion
product was treated with 15 units of mung bean nuclease
to change cohesive ends to blunt ends. The DNA fragment
thus-obtained was further digested with BglII, and after
agarose gel electrophoresis, a DNA fragment of about
10 500 bp was isolated. On the other hand, plasmid
p6huγN1-CKpn was digested with KpnI, the cohesive ends
were changed to blunt ends using T4 DNA polymerase, and
after digestion with BamHI followed by agarose gel
electrophoresis, a DNA fragment of about 4800 bp was
15 obtained. The DNA fragments thus-prepared were mixed
and ligated using T4 DNA ligase, and the reaction
mixture was used to transform E. coli HB101 (Boyer et
al., J. Mol. Biol., 41, 459-472, 1969). The resulting
ampicillin resistant transformants were subjected to
20 colony hybridization using as a probe a 32P-labeled DNA
fragment prepared by nick-translating a ClaI-BglII
fragment of plasmid pKM6, to obtain 6 positive clones
among 56 clones. Plasmid DNA was extracted from each of
these clones, and a restriction enzyme cleavage map was
25 made. As a result, it was confirmed that plasmids from
all positive clones had a structure as shown in Fig. 8.
Moreover, plasmid DNA derived from a representative
clone γβ6 was digested with SalI, and resulting DNA
fragments were inserted to M13 phage to determine a
30 nucleotide sequence of the plasmid DNA. As a result, in
this plasmid, an IFN-γ structural gene was linked with
an IFN-β structural gene in frame. This desired plasmid
was designated as plasmid ptrp6huIFN-γβ, and a trans-
formant E. coli was designated as E. coli HB101
35 (ptrp6huIFN-γβ).

Example 2. Construction of plasmid ptrp6huIFN-βα
for expression of interferon-β·α

conjugate

A process f r the construction of a plasmid
ptrp6huIFN-βγ is shown in Fig. 9, wherein 20 μg f
plasmid pKM6-Cxho was digested with Xho I, and the

5 digestion product was treated with 15 units of mung bean
nuclease at 37°C for 15 minutes to make blunt ends, and
then digested with SalI. The reaction mixture was
subjected to agarose gel electrophoresis to isolate a
DNA fragment of about 4500 bp. On the other hand, 30 μg

10 of p6huγN1ΔBS-NHin was digested with HinPI, the digestion
product was treated with 30 units of mung bean nuclease
at 37°C for 15 minutes, and digested with SalI, and
after agarose gel electrophoresis a DNA fragment of
about 860 bp was isolated.

15 The DNA fragments thus-prepared were mixed and
ligated using the T4 DNA ligase, and the reaction
mixture was used to transform E. coli HB101. Among the
resulting ampicillin resistant transformants, 50 clones
were subjected to colony hybridization using a

20 oligonucleotide probe 5'-AGTCAGATGCTGTTTC-3', which is
the same oligonucleotide used for the construction of
plasmid p6huγN1-CKpn, to obtain 28 positive clones.
A plasmid DNA was isolated from a representative
clone βγ31, and a restriction enzyme cleavage map was

25 made. As a result, a map as shown in Fig. 9 was
obtained. Moreover, a BstEII-SalI fragment from the
plasmid isolated from the clone βγ31 was cloned in phage
M13 to determine the nucleotide sequence. As a result,
in the plasmid, an IFN-β structural gene was linked with

30 an IFN-γ structural gene in frame. This plasmid was
designated as plasmid ptrp6huIFN-βγ, and E. coli
transformed with the plasmid was designated as E. coli
HB101 (ptrp6huIFN-βγ).

Example 3. Construction of plasmid ptrphuIFN-γcβ

35 for expression of interferon-γcβ

conjugate

A process for the construction of a ptrphuIFN-γcβ

is shown in Fig. 10. A plasmid pKM6 was digested with
ClaI and then with BglII, and after agaros gel
electrophoresis a DNA fragment of about 500 bp was
isolated. On the other hand, a DNA fragment coding for
5   a spacer peptide was prepared from four synthetic
oligonucleotides according to the same procedure as
described in paragraph (2) of the Reference Example. 10
pmoles of this DNA fragment was mixed with the ClaI-BglII
fragment of pKM6 isolated as described above and the DNA
10   fragment of about 4800 bp derived from plasmid
p6huγNl-CKpn as described in Example 1, these DNA
fragments were ligated using the T4 DNA ligase, and the
reaction mixture was used to transform E. coli HB101.
82 clones of the resulting ampicillin resistant trans-
15   formants were subjected to colony hybridization using
the probe described in Example 1 to obtain three positive
clones. Plasmid DNAs were isolated from the three
clones, and restriction enzyme cleavage maps were made.
As a result, only one clone contained a plasmid having a
20   desired structure. This plasmid was designated as
plasmid ptrp6huIFN-γcβ, and E. coli transformed with
this plasmid was designated as E. coli HB101
(ptrp6huIFN-γcβ).

Example 4. Culturing and production of interferon
25   conjugate

Transformants obtained in Examples 1 to 3 were
inoculated to LB medium (1.0% bactotrypton, 0.5% yeast
extract, 0.5% sodium chloride, 0.1% glucose, pH 7.2 with
sodium hydroxide) supplemented with 100 µg/ml tryptophan,
30   and 100 µg/ml ampicillin, and cultivation was carried
out at 30°C for 8 hours. This culture was then
inoculated to M9 medium (0.3% mono potassium phosphate,
0.6% disodium phosphate, 0.1% ammonium chloride, and
0.5% sodium chloride, and further, separately sterilized
35   1 µg/ml Vitamine B_1 and 0.1 mM magnesium sulfate)
supplemented with 1.0% glucose and 1.0% casamino acids,
in an amount of 10% by volume relating to a volume of

the M9 medium, and cultivation was continued. After
about 10 hours, ind leacrylic acid was add d to the
final c nc ntrati n f 10 μg/ml, and cultivation was
carried out for an additi nal 8 hours. During the
cultivation, if necessary, 40% glucose aqueous solution
was added to the culture medium to prevent depletion of
the glucose. Moreover, during the cultivation, a 14%
aqueous ammonia solution was added to the culture medium
to maintain the pH value at 6.0 to 7.0.

After the cultivation, 2 ml of the cultured broth
was centrifuged at 10,000X g for 4 minutes to collect
cells. The cells were washed with physiological saline,
and the washed cells were suspended in 1 ml of 50 mM
Tris-HCl buffer (pH 7.5) containing 3 mg of lysozyme,
2 mM EDTA, 30 mM sodium chloride, and 20% glycerol,
and the suspension was allowed to stand in ice for
60 minutes. The freezing and thawing of the suspension
were repeated three times to disrupt the cells, and the
disrupted mixture was centrifuged at 30,000X g for
20 minutes to eliminate cell debris. A supernatant thus
obtained was used as a test sample for measuring.

The antiviral activity of interferon was measured
according to "Interferon no Kagaku" (Science of
Interferon) edited by Shigeyasu Kobayashi, p13-20, Kodan
Sha, 1985, by the CPE_{50} inhibition method using the FL
cells-sindbis virus system. As a standard for
measurement of the activity, an IFN-γ preparation was
used which was produced by recombinant cells and
calibrated using NIH natural IFN-γ Gg23-901-530. For
reference, interferon crude extracts prepared according
to the above-mentioned procedure from E. coli HB101
containing plasmid pKM6, which expresses human
interferon-β, and from E. coli HB101 containing plasmid
p6hur-N1, which express human interferon-γ, were tested
for their antiviral activity. The results are shown in
Table 1.

Table 1

Strain	Antiviral Activity per Extract (U/ml)
E. coli HB101 (ptrp6huIFN-γβ)	3.9×10^4
E. coli HB101 (ptrp6huIFN-βγ)	1.6×10^4
E. coli HB101 (ptrp6huIFN-γcβ)	7.7×10^4
E. coli HB101 (pKM6)	3.1×10^5
E. coli HB101 (p6huγ-N1)	4.1×10^4

As seen from Table 1, each preparation exhibited an
20 antiviral activity characteristic for the expected
interferon.

Example 5. Measurement of Molecular Weight

1 ml of cultured broth prepared according to the
same procedure as described in Example 4 was centrifuged
25 at 10,000X g for 4 minutes to collect cells. The cells
were suspended in 500 μl of 62.5 mM Tris-HCl buffer
(pH 6.8) containing 5% 2-mercaptoethanol and 2% sodium
dodecylsulfate, and the suspension was heated in a
boiling water bath for 5 minutes and allowed to cool.
30 Then, to the heat-treated suspension was added 50 μl of
62.5 mM Tris-HCl buffer (pH 6.8) containing 0.05% bromo
phenol blue and 70% glycerol, to prepare a sample for
electrophoresis. SDS-polyacrylamide gel electrophoresis
was carried out according to Laemmli's method (Nature,
35 227, 680, 1970). The gel concentration was 15%. As
maker proteins, lysozyme having a molecular weight of
14,400, trypsin inhibitor having a molecular weight of

21,500, carbonic anhydras having a mol cular weight f
31,000, ovalbumin having a mol cular w ight f 45,000,
bovine serum albumin having a molecular weight of
66,200, and phosphorylase B having a molecular weight of
5 92,500 were used. After electrophoresis, the gel was
stained with Coomasie Brilliant Blue R250 to detect
proteins. Another gel obtained by simultaneous electro-
phoresis was used to transport proteins on the gel to a
nitrocellulose sheet. The nitrocellulose sheet was
10 reacted with commercially available anti-human
interferon-β immunoglobulin or anti-human interferon-γ
immunoglobulin, and then reacted with peroxidase-labeled
protein A to determine the position of the interferon
conjugate. By comparing a result of the above-mentioned
15 Westean blotting with the positions of electro-
phoretically migrated maker proteins, it was found that
IFN-γβ and IFN-βγ have a molecular weight of about
37,000, and IFN-γcβ has a molecular weight of about
38,000. That is, it was confirmed that interferon
20 conjugates of the present invention substantially
consist of human interferon-β having a molecular weight
of about 20,000 and human interferon-γ having a molecular
weight of about 17,000. Note, about 1000 of a molecular
weight in IFN-γcβ is assigned to a spacer peptide
25 contained in the IFN-γcβ.

Example 6. Neutralization of product with antibody

A crude interferon extract from the E. coli HB101
(ptrp6huIFN-γβ) prepared according to the procedure
described in Example 4 was diluted five-fold with an
30 Eagle's minimum essential medium containing 5% calf
serum and 10 mM Hepes (pH 7.3). To 1 ml of the
interferon solution thus-prepared, 1 ml of anti-IFN-β
rabbit antiserum diluted 50-fold with the same medium
(neutralization value 2700 U/ml) or 1 ml of anti-IFN-γ
35 rabbit antiserum diluted 50-fold with the same medium
(neutralization value 2000 U/ml) was added, and after
the mixture was incubated at 37°C for 30 minutes, the

antiviral activity was measured. For control, a reaction
mixture wherein the antiserum was replaced by the
medium, and a reaction mixture wherein 0.5 ml of the
anti-IFN-β rabbit antiserum solution and 0.5 ml of the
5   anti-IFN-γ rabbit antiserum solution were added were
prepared, and the antiviral activity was measured in
same manner. The results are set forth in Table 2.

Table 2

Antiserum	Antiviral Activity (U/ml)
Control (no antiserum)	6.0×10^3
Anti-IFN-β antiserum	1.3×10^3
Anti-IFN-γ antiserum	1.7×10^3
Anti-IFN-β antiserum + and -IFN-γ antiserum	8.1×10

As seen from Table 2, each of the anti-IFN-β
antiserum and the anti-IFN-γ antiserum partially
neutralized antiviral activity, and a combination
of anti-IFN-β antiserum and anti-IFN-γ antiserum
25   substantially completely neutralized all antiviral
activity. This means that the present interferon-γβ
conjugate has both an interferon-β action and an
interferon-γ action. Moreover, although 2.7×10^3 U of
anti-IFN-β antiserum neutralized the IFN-β activity with
30   1.3×10^3 U of IFN-γ activity remaining and 2.0×10^3 U
of anti-IFN-γ antiserum neutralized the IFN-γ activity
with 1.7×10^3 U of IFN-β activity remaining, the
not-neutralized control showed 6.0×10^3 U activity.
That is, antiviral activity of the control (6.0×10^3)
35   is higher than total amount of remaining activities ($1.3
\times 10^3$ U + 1.7×10^3 U). This means that the present
interferon-γβ conjugate exhibits synergism of IFN-β

activity and IFN-γ activity.

Example 7. Construction of plasmid ptrp6huIFN-βcγ for expression of interferon βcγ

A process for the construction of a plasmid ptrp6huIFN-βcγ is shown in Fig. 12, wherein 20 μg of plasmid pKM6-Cxho was digested with XhoI, the digestion product was treated with 15 units of mung been nuclease at 37°C for 15 minutes to make blunt ends, and then digested with SalI. The reaction mixture was subjected to agarose gel electrophoresis to isolate a DNA fragment of about 4500 bp. On the other hand, 30 μg of plasmid p6huγN1ΔBS-NHin was digested with HinPI and SalI, and after agarose gel electrophoresis, a DNA fragment of about 860 bp was isolated. The above-mentioned two DNA fragments were mixed with 10 pmoles of DNA fragment coding for a spacer peptide obtained according to the procedure as described in Example 3, these three DNA fragments were ligated with T4 DNA ligase, and the ligation mixture was used to transform E. coli HB101. The 204 ampicillin resistant transformants thus obtained were subjected to colony hybridization using as a probe that shown in Example 2 or an oligonucleotide 5'-CGTTACCGACTTAGCA-3' used for preparation of the DNA fragment coding for the spacer peptide. As a result, two clones were found to be positive, and plasmids from these clones were analyzed by restriction enzymes. One clone contained a desired plasmid, and this plasmid was designated as plasmid ptrp6huIFN-βcγ, and E. coli transformed with this plasmid was designated as E. coli HB101 (ptrp6huIFN-βcγ). According to the procedures described in Example 4, the E. coli transformant was cultured and an extract prepared, and then the antiviral activity was measured. An antiviral activity of 3.9 x 10^4 U/ml extract was found.

Example 8. Neutralization of product with antibody

According to the same procedure as described in Example 6, a crude interferon extract from E. coli HB101

(ptrp6huIFN-γcβ) was neutralized with antisera. For comparison, an interf ron mixture consisting of 8600 U/ml of IFN-β and 2400 U/ml of IFN-γ, prepared by mixing an IFN-β and an IFN-γ in approximately the same molecular

5 numbers, were tested in the same manner. The results are shown in Table 3.

Table 3

IFN	Antiserum		Antiviral activity
	Anti-IFN-β	Anti-IFN-γ	(U/ml)
IFN mixture	–	–	19000
	o	–	930
	–	o	12000
	o	o	<27
IFN-γcβ	–	–	22000
	o	–	2400
	–	o	11000
	o	o	61

As can be seen from Table 3, in the interferon-γcβ conjugate (IFN-γcβ), each of the anti-IFN-β antiserum and anti-IFN-γ antiserum partially neutralized the antiviral activity thereof, and a combination of both

30 antisera substantially completely neutralized all antiviral activity. This means that correctly folded IFN-β and IFN-γ molecules are liked to yield both the IFN-β activity and IFN-γ activity are exhibited by a single polypeptide.

35 Moreover, from the comparison of the activities resulting from neutralization with the anti-IFN-β or anti-IFN-γ with the activity resulting from

neutr lization with f a combination of anti-IFN-β
and anti-IFN-γ, it appears that the present IFN-γcβ
conjugat als exhibits the synergism of the IFN-β
activity and the IFN-γ activity.

5   Example· 9.

     A.   Construction of vector for expression of human
          interferon-β

          pSVβ is a vector derived from the human
interferon-γ expression vector pSV2IFNβ (Japanese
10   Unexamined Patent Publication No. 61-52283) by deleting
a sequence inhibiting replication of the vector in a
eukaryotic cell (M. Losky et al., Nature, 293 77, 1981).
The vector pSVβ was constructed as follows.

          First, a PvuII site positioned in the pSV2IFNβ
15   upstream from the SV40 early promoter was replaced by a
SalI site using a SalI linker, and the modified vector
was cleaved with SalI and BamHI to isolate a 1.7 Kb DNA
fragment required for expression of the human
interferon-β.

20   Next, vector pML2d (M. Lusky et al., Nature,
293, 79, 1981) derived from pBR322 by deleting a sequence
inhibiting replication of the vector in the eukaryotic
cell was cleaved with SalI and BamHI to isolate a long
DNA fragment.

25   These two DNA fragments were ligated using T4
DNA ligase to obtain pSVβ.

     B.   Construction of vector pMTVβ for expression of
          human interferon-β

          The vector pSVβ constructed in Section A was
30   cleaved with SalI, and the SalI site in the vector was
replaced by a HindIII site using a HindIII linker, and
the modified vector was cleaved with HindIII to isolate
a 3.8 Kb DNA fragment which does not contain the SV40
early promoter. The DNA fragment thus obtained was then
35   treated with E. coli alkaline phosphatase to eliminate a
terminal phosphate group.

          Next, vector pMTVdhfr (F. Lee et al., Nature,

294, 228, 1982) containing MMTV promoter was cleav d
with a restriction enzyme HindIII to isolate a 1.4 Kb
DNA fragment containing MMTV promoter.

These two DNA fragments wer ligated using T4
5 DNA ligase to obtain pMTVβ.

C.   Construction of vector pMTVγ for expression of
      human interferon-γ

pMTVγ is a vector wherein a human interferon-γ
gene is positioned under control by an MMTV promoter.
10   The vector pMTVβ constructed in Section B was
cleaved at a HindIII site downstream from the MMTV
promoter and at the BglII site downstream from the human
interferon gene, and after the resulting fragments were
blunt-ended using a DNA polymerase I Klenow fragment, a
15 3.9 Kb DNA fragment containing the MMTV promoter was
isolated.

This DNA fragment was ligated with a 0.8 Kb
DNA fragment containing a human interferon-γ gene, which
was obtained from pSVIFNγ (Japanese Unexamined Patent
20 Publication No. 61-52286) by cleaving with DpnI, using a
T4 DNA ligase to obtain pMTVγ.

D.   Construction of vector pMTV(SV)γ for expression
      of human interferon-γ

pMTV(SV)γ is a vector derived from pMTVγ by
25 inserting an SV40 early promoter upstream of the MMTV
promoter. The vector pMTV(SV)γ was constructed as
follows.

pMTVγ constructed in Section C was cleaved
with SalI, and after the resulting DNA fragment was
30 blunt-ended using a DNA polymerase I Klenow fragment, a
terminal phosphate group was eliminated by BAP treatment.

Next, pSV2IFNβ (Japanese Unexamined Patent
Publication 61-52283) was cleaved with PvuII and HindIII
to isolate a 0.3 Kb DNA fragment containing the SV40
35 early promoter, and the fragment was blunt-ended by
treating with a DNA polymerase I Klenow fragment.

These two DNA fragments were ligated using T4

DNA ligase t obtain pMTV(SV)γ.

E. Construction of plasmid pMTV(SV)γ·β f r
    expr ssi n of human interferon-γ·β conjugate
    in animal cells (Fig. 13)

5   pMTV(SV)γ·β is a vector wherein the human
interferon-γ gene in pMTV(SV)γ has been replaced by the
human interferon-γ·β conjugate gene, and was constructed
as follows.

        10 μg of ptrp6huIFN-γβ constructed according
10 to the procedure as described in Example 1 was digested
with NdeI and DpnI, and the digestion mixture was
subjected to agarose gel electrophoresis to isolate a
DNA fragment of about 1300 bp.

        On the other hand, pMTV(SV)γ constructed in
15 Section D was digested with BglII, and the resulting
fragment was blund-ended by treating with DNA
polymerase I Klenow fragment, followed by digestion with
NdeI, and the reaction mixture was subjected to agarose
gel electrophoresis to isolate a DNA fragment of about
20 5100 bp.

        These DNA fragments were mixed and ligated
with T4 DNA ligase to obtain pMTV(SV)γ·β.

        Example 10. Construction of plasmid pMTV(SV)γcβ
                     for expression of human interferon-γcβ
25   conjugate in animal cells

        Plasmid pMTV(SV)γcβ is a vector wherein a human
interferon-γ gene in pMTV(SV)γ has been replaced by a
human interferon-γcβ conjugate gene, and was constructed
as follows.

30   10 μg of ptrp6huIFN-γcβ constructed according to
the procedure as described in Example 3 was digested
with NdeI and DpnI, and the reaction mixture was
subjected to agarose gel electrophoresis to isolate a
DNA fragment of about 1300 bp. On the other hand,
35 pMTV(SV)γ constructed in Section D was digested with
BglII, and the resulting DNA fragment was blunt-ended by
treating with a DNA polymerase I Klenow fragment,

followed by digestion with NdeI, and the reaction
mixture was subjected to agarose gel electrophoresis to
isolate a DNA fragment of about 5100 bp.

These DNA fragments were mixed, and ligated using
T4 DNA ligase to obtain pMTV(SV)γcβ.

Example 11. Transformation of PC12 cell with
MTV(SV)γ·β

4 µg of vector pMTV(SV)γ·β constructed in Example 9
and 0.4 µg of GC18 resistance gene expression vector
pSV2neo (J. Southern et al., J. Mo. Appl. Genet.,$\underline{1}$,
327, 1982) were introduced into about 10^6 cells of PC12
cell line (M. Kinjo et al., Br. J. Cancer, $\underline{39}$, 15, 1979)
derived from human lung cancer, according to the calcium
phosphate method. The cells thus-prepared were cultured
in a selective medium (RPMI 1640 medium supplemented
with 10% fetal calf serum and 100 µg/ml kanamycine;
Nissui Seiyaku, Japan) containing 400 µg/ml of a protein
synthesis inhibitor, GC18 (GIBCO) to obtain 24 trans-
formant clones. The antiviral activity of the cultured
supernatants was tested using FL cells-sindbis virus
system according to the CPE_{50} inhibition method described
in Example 4. As a result, supernatants from 22 clones
exhibited the antiviral activity, as shown in Table 4.

0237019

Table 4

pMTV(SV)γ·β/PC12	
Clone	Antiviral activity (U/ml)
1	18500
2	1100
3	600
4	1500
5	< 80
6	1300
7	200
8	2300
9	200
10	< 80
11	1000
12	2500
13	900
14	1400
15	500
16	400
17	< 80
18	< 80
19	300
20	800
21	200
22	300
23	200
24	600

Example 12. Transformati n of PC12 cells with
pMTV(SV)γcβ

4 µg f pMTV(SV)γcβ constructed in Example 10 and
0.4 µg of pSV2neo (see Example 11) were introduced into
5   about 10^6 cells of the PC12 cell line by a calcium
phosphate method according to the same procedure as
described in Example 11. The cells thus-prepared were
cultured in a selective medium (RPMI 1640 medium
supplemented with 10% fetal calf serum and 100 µg/ml
10   kanamycine; Nissui Seiyaku, Japan) to obtain 26
transformed clones. The antiviral activity of the
cultured supernatants was tested by the CPE_{50} inhibition
method using the FL cells-sindbis virus system according
to the same procedure as described in Example 11. As a
15   result, supernatants from 20 clones exhibited the
antiviral activity as shown in Table 5.

Table 5

pMTV(SV)γcβ/PC12	
Clone	Antiviral activity (U/ml)
1	400
2	< 60
3	< 60
4	3800
5	1200
6	< 60
7	6400
8	200
9	< 80
10	400
11	200
12	900
13	500
14	700
15	< 80
16	1300
17	21450
18	130
19	5300
20	1600
21	200
22	< 80
23	1200
24	8600
25	300
26	500

CLAIMS

1. An interferon conjugate c mprising in a single
m lecule a region exhibiting biological activities f
interferon-β and a region exhibiting biological
activities of interferon-γ.

5   2.   An interferon conjugate according to claim 1,
which is a biologically active polypeptide having no
glycoside chain.

3.   An interferon conjugate according to claim 1,
which is a biologically active polypeptide having
10 glycoside chains.

4.   An interferon conjugate according to any
one of claims 1 to 3, wherein the region exhibiting
biological activities of interferon-β and the region
exhibiting biological activities of interferon-γ have
15 been linked via a spacer.

5.   An interferon conjugate according to claim 4,
wherein the spacer is a polypeptide.

6.   An interferon conjugate according to any one
of claims 1 to 3, wherein a C-terminal of the region
20 exhibiting biological activities of interferon-β has
been linked to an N-terminal of the region exhibiting
biological activities of interferon-γ, directly or via a
spacer.

7.   An interferon conjugate according to claim 6,
25 wherein the interferon-β consists of the following amino
acid sequence:

MET SER TYR ASN LEU LEU GLY PHE LEU GLN ARG SER SER
ASN PHE GLN CYS GLN LYS LEU LEU TRP GLN LEU ASN GLY ARG LEU GLU TYR
CYS LEU LYS ASP ARG MET ASN PHE ASP ILE PRO GLU GLU ILE LYS GLN LEU
GLN GLN PHE GLN LYS GLU ASP ALA ALA LEU THR ILE TYR GLU MET LEU GLN
ASN ILE PHE ALA ILE PHE ARG GLN ASP SER SER SER THR GLY TRP ASN GLU
THR ILE VAL GLU ASN LEU LEU ALA ASN VAL TYR HIS GLN ILE ASN HIS LEU
LYS THR VAL LEU GLU GLU LYS LEU GLU LYS GLU ASP PHE THR ARG GLY LYS
LEU MET SER SER LEU HIS LEU LYS ARG TYR TYR GLY ARG ILE LEU HIS TYR
LEU LYS ALA LYS GLU TYR SER HIS CYS ALA TRP THR ILE VAL ARG VAL GLU
ILE LEU ARG ASN PHE TYR PHE ILE ASN ARG LEU THR GLY TYR LEU ARG ASN,

and the interferon-γ consists of the following amino
acid sequence:

GLN ASP PRO TYR VAL LYS GLU ALA GLU ASN LEU LYS LYS
TYR PHE ASN ALA GLY HIS SER ASP VAL ALA ASP ASN GLY THR LEU PHE LEU
GLY ILE LEU LYS ASN TRP LYS GLU GLU SER ASP ARG LYS ILE MET GLN SER
GLN ILE VAL SER PHE TYR PHE LYS LEU PHE LYS ASN PHE LYS ASP ASP GLN
SER ILE GLN LYS SER VAL GLU THR ILE LYS GLU ASP MET ASN VAL LYS PHE
PHE ASN SER ASN LYS LYS LYS ARG ASP ASP PHE GLU LYS LEU THR ASN TYR
SER VAL THR ASP LEU ASN VAL GLN ARG LYS ALA ILE HIS GLU LEU ILE GLN
VAL MET ALA GLU LEU SER PRO ALA ALA LYS THR GLY LYS ARG LYS ARG SER
GLN MET LEU PHE ARG GLY ARG ARG ALA SER GLN.

8.   An interferon conjugate according to claim 7,
wherein a C-terminal of a polypeptide consisting of the
following amino acid sequence:

MET SER TYR ASN LEU LEU GLY PHE LEU GLN ARG SER SER
ASN PHE GLN CYS GLN LYS LEU LEU TRP GLN LEU ASN GLY ARG LEU GLU TYR
CYS LEU LYS ASP ARG MET ASN PHE ASP ILE PRO GLU GLU ILE LYS GLN LEU
GLN GLN PHE GLN LYS GLU ASP ALA ALA LEU THR ILE TYR GLU MET LEU GLN
ASN ILE PHE ALA ILE PHE ARG GLN ASP SER SER SER THR GLY TRP ASN GLU
THR ILE VAL GLU ASN LEU LEU ALA ASN VAL TYR HIS GLN ILE ASN HIS LEU
LYS THR VAL LEU GLU GLU LYS LEU GLU LYS GLU ASP PHE THR ARG GLY LYS
LEU MET SER SER LEU HIS LEU LYS ARG TYR TYR GLY ARG ILE LEU HIS TYR
LEU LYS ALA LYS GLU TYR SER HIS CYS ALA TRP THR ILE VAL ARG VAL GLU
ILE LEU ARG ASN PHE TYR PHE ILE ASN ARG LEU THR GLY TYR LEU ARG ASN
has been linked to an N-terminal of a polypeptide
consisting of the following amino acid sequence:

GLN ASP PRO TYR VAL LYS GLU ALA GLU ASN LEU LYS LYS
TYR PHE ASN ALA GLY HIS SER ASP VAL ALA ASP ASN GLY THR LEU PHE LEU
GLY ILE LEU LYS ASN TRP LYS GLU GLU SER ASP ARG LYS ILE MET GLN SER
GLN ILE VAL SER PHE TYR PHE LYS LEU PHE LYS ASN PHE LYS ASP ASP GLN
SER ILE GLN LYS SER VAL GLU THR ILE LYS GLU ASP MET ASN VAL LYS PHE
PHE ASN SER ASN LYS LYS LYS ARG ASP ASP PHE GLU LYS LEU THR ASN TYR
SER VAL THR ASP LEU ASN VAL GLN ARG LYS ALA ILE HIS GLU LEU ILE GLN
VAL MET ALA GLU LEU SER PRO ALA ALA LYS THR GLY LYS ARG LYS ARG SER
GLN MET LEU PHE ARG GLY ARG ARG ALA SER GLN,
via a polypeptide as a spacer.

9.   An interferon conjugate according to any one

of claims 1 to 3, wherein a C-terminal f the regi n
exhibiting biological activities of interfer n-γ has
be n linked to an N-terminal of the region exhibiting
biological activities of interferon-β, directly or via a
5   spacer.

10.   An interferon conjugate according to claim 9,
wherein the interferon-γ consists of the following amino
acid sequence:

GLN ASP PRO TYR VAL LYS GLU ALA GLU ASN LEU LYS LYS
TYR PHE ASN ALA GLY HIS SER ASP VAL ALA ASP ASN GLY THR LEU PHE LEU
GLY ILE LEU LYS ASN TRP LYS GLU GLU SER ASP ARG LYS ILE MET GLN SER
GLN ILE VAL SER PHE TYR PHE LYS LEU PHE LYS ASN PHE LYS ASP ASP GLN
SER ILE GLN LYS SER VAL GLU THR ILE LYS GLU ASP MET ASN VAL LYS PHE
PHE ASN SER ASN LYS LYS LYS ARG ASP ASP PHE GLU LYS LEU THR ASN TYR
SER VAL THR ASP LEU ASN VAL GLN ARG LYS ALA ILE HIS GLU LEU ILE GLN
VAL MET ALA GLU LEU SER PRO ALA ALA LYS THR GLY LYS ARG LYS ARG SER
GLN MET LEU PHE ARG GLY ARG ARG ALA SER GLN,
and the interferon-β consists of the following amino
acid sequence:

MET SER TYR ASN LEU LEU GLY PHE LEU GLN ARG SER SER
ASN PHE GLN CYS GLN LYS LEU LEU TRP GLN LEU ASN GLY ARG LEU GLU TYR
CYS LEU LYS ASP ARG MET ASN PHE ASP ILE PRO GLU GLU ILE LYS GLN LEU
GLN GLN PHE GLN LYS GLU ASP ALA ALA LEU THR ILE TYR GLU MET LEU GLN
ASN ILE PHE ALA ILE PHE ARG GLN ASP SER SER SER THR GLY TRP ASN GLU
THR ILE VAL GLU ASN LEU LEU ALA ASN VAL TYR HIS GLN ILE ASN HIS LEU
LYS THR VAL LEU GLU GLU LYS LEU GLU LYS GLU ASP PHE THR ARG GLY LYS
LEU MET SER SER LEU HIS LEU LYS ARG TYR TYR GLY ARG ILE LEU HIS TYR
LEU LYS ALA LYS GLU TYR SER HIS CYS ALA TRP THR ILE VAL ARG VAL GLU
ILE LEU ARG ASN PHE TYR PHE ILE ASN ARG LEU THR GLY TYR LEU ARG ASN.

30   11.   An interferon conjugate according to claim 10,
wherein a C-terminal of a polypeptide consisting of the
following amino acid sequence:

GLN ASP PRO TYR VAL LYS GLU ALA GLU ASN LEU LYS LYS
TYR PHE ASN ALA GLY HIS SER ASP VAL ALA ASP ASN GLY THR LEU PHE LEU
GLY ILE LEU LYS ASN TRP LYS GLU GLU SER ASP ARG LYS ILE MET GLN SER
GLN ILE VAL SER PHE TYR PHE LYS LEU PHE LYS ASN PHE LYS ASP ASP GLN
SER ILE GLN LYS SER VAL GLU THR ILE LYS GLU ASP MET ASN VAL LYS PHE

PHE ASN SER ASN LYS LYS LYS ARG ASP ASP PHE GLU LYS LEU THR ASN TYR

SER VAL THR ASP LEU ASN VAL GLN ARG LYS ALA ILE HIS GLU LEU ILE GLN

VAL MET ALA GLU LEU SER PRO ALA ALA LYS THR GLY LYS ARG LYS ARG SER

GLN MET LEU PHE ARG GLY ARG ARG ALA SER GLN

has been linked to an N-terminal of a polypeptide
consisting of the following amino acid sequence:

MET SER TYR ASN LEU LEU GLY PHE LEU GLN ARG SER SER

ASN PHE GLN CYS GLN LYS LEU LEU TRP GLN LEU ASN GLY ARG LEU GLU TYR

CYS LEU LYS ASP ARG MET ASN PHE ASP ILE PRO GLU GLU ILE LYS GLN LEU

GLN GLN PHE GLN LYS GLU ASP ALA ALA LEU THR ILE TYR GLU MET LEU GLN

ASN ILE PHE ALA ILE PHE ARG GLN ASP SER SER SER THR GLY TRP ASN GLU

THR ILE VAL GLU ASN LEU LEU ALA ASN VAL TYR HIS GLN ILE ASN HIS LEU

LYS THR VAL LEU GLU GLU LYS LEU GLU LYS GLU ASP PHE THR ARG GLY LYS

LEU MET SER SER LEU HIS LEU LYS ARG TYR TYR GLY ARG ILE LEU HIS TYR

LEU LYS ALA LYS GLU TYR SER HIS CYS ALA TRP THR ILE VAL ARG VAL GLU

ILE LEU ARG ASN PHE TYR PHE ILE ASN ARG LEU THR GLY TYR LEU ARG ASN,

via a polypeptide as a spacer

12. A deoxyribonucleotide sequence coding for an
interferon conjugate comprising in a single molecule a
20   region exhibiting biological activities of interferon-β
and a region exhibiting biological activities of
interferon-γ.

13. A recombinant DNA comprising a deoxyribon-
ucleotide sequence coding for an interferon conjugate
25   comprising in a single molecule a region exhibiting
biological activities of interferon-β and a region
exhibiting biological activities of interferon-γ, and a
deoxyribonucleotide sequence coding for a control region
for expression of said interferon conjugate, wherein the
30   latter sequence is upstream from the former sequence.

14. A transformant organism transformed with a
recombinant DNA comprising a deoxyribonucleotide sequence
coding for an interferon conjugate comprising in a
single molecule a region exhibiting biological activities
35   of interferon-β and a region exhibiting biological
activities of interferon-γ, and a deoxyribonucleotide
sequence coding for a control region for expression of

said interferon conjugat , wherein the latter sequence
is upstream from the former s quence.

    15.   A process for production of an interf r n
conjugate comprising in a single molecule a r gion
5 exhibiting biological activities of interferon-β and a
region exhibiting biological activities of interferon-β,
comprising the step:

        culturing a transformant organism trans-
formed with a recombinant DNA comprising a deoxyribon-
.10 ucleotide sequence coding for said interferon conjugate
and a deoxyribonucleotide sequence coding for a control
region for expression of the interferon conjugate
wherein the latter sequence is upstream from the former
sequence, to produce the interferon conjugate;

15   and recovering said interferon conjugate.

    16.   A process according to claim 15, wherein E.
coli is used as a host.

    17. . A process according to claim 11, wherein human
cells are used as a host.

20   18.   A process according to claim 17, wherein the
human cells are cells derived from human lung cancer.

    19.   A process according to claim 18, wherein the
cells derived from human lung cancer are selected from
the group consisting of PC8 and PC12 cell lines.

Fig. I

```
                                                          MET SER TYR ASN

LEU LEU GLY PHE LEU GLN ARG SER SER ASN PHE GLN CYS GLN LYS LEU LEU TRP GLN LEU ASN GLY ARG LEU GLU

TYR CYS LEU LYS ASP ARG MET ASN PHE ASP ILE PRO GLU GLU ILE LYS GLN LEU GLN GLN PHE GLN LYS GLU ASP

ALA ALA LEU THR ILE TYR GLU MET LEU GLN ASN ILE PHE ALA ILE PHE ARG GLN ASP SER SER SER THR GLY TRP

ASN GLU THR ILE VAL GLU ASN LEU LEU ALA ASN VAL TYR HIS GLN ILE ASN HIS LEU LYS THR VAL LEU GLU GLU

LYS LEU GLU LYS GLU ASP PHE THR ARG GLY LYS LEU MET SER SER LEU HIS LEU LYS ARG TYR TYR GLY ARG ILE

LEU HIS TYR LEU LYS ALA LYS GLU TYR SER HIS CYS ALA TRP THR ILE VAL ARG VAL GLU ILE LEU ARG ASN PHE

TYR PHE ILE ASN ARG LEU THR GLY TYR LEU ARG ASN
```

Fig. 2

GLY ASP PRO TYR VAL LYS GLU ALA GLU ASN LEU LYS LYS TYR PHE ASN ALA GLY HIS SER ASP VAL

ALA ASP ASN GLY THR LEU PHE LEU GLY ILE LEU LYS ASN TRP LYS GLU GLU SER ASP ARG LYS ILE MET GLN SER

GLN ILE VAL SER PHE TYR PHE LYS LEU PHE LYS ASN PHE LYS ASP ASP GLN SER ILE GLN LYS SER VAL GLU THR

ILE LYS GLU ASP MET ASN VAL LYS PHE PHE ASN SER ASN LYS LYS ARG ASP ASP PHE GLU LYS LEU THR ASN

TYR SER VAL THR ASP LEU ASN VAL GLN ARG LYS ALA ILE HIS GLU LEU ILE GLN VAL MET ALA GLU LEU SER PRO

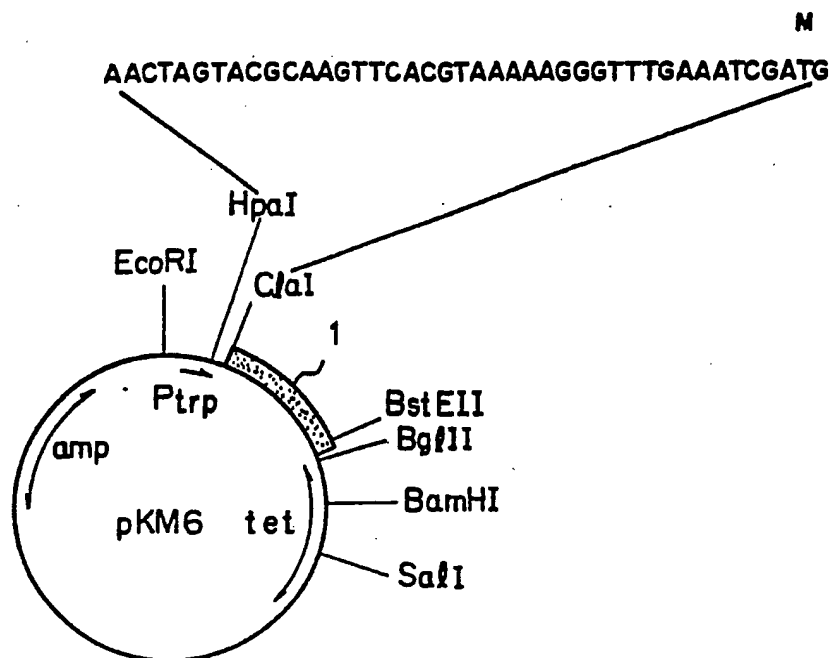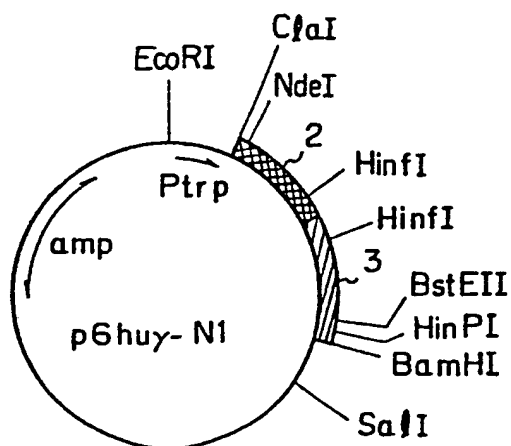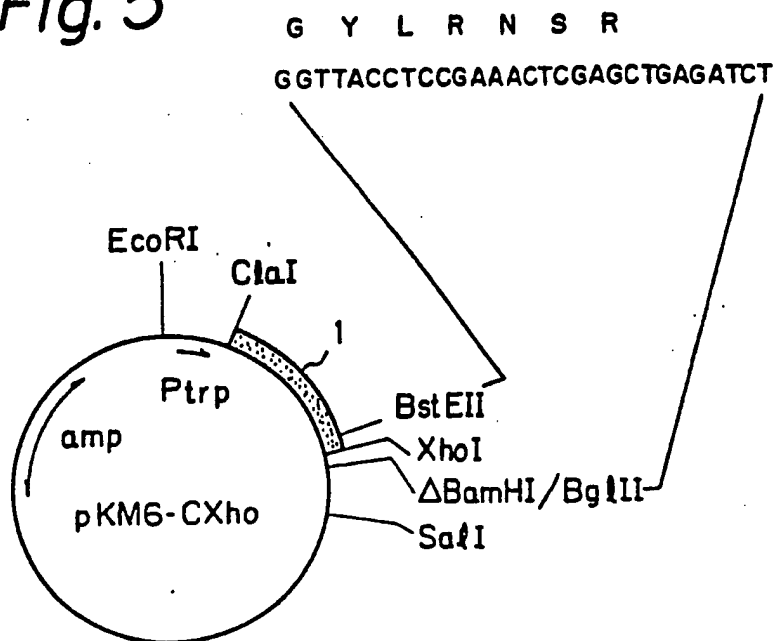ALA ALA LYS THR GLY LYS ARG LYS ARG SER GLN MET LEU PHE ARG GLY ARG ARG ALA SER GLN

Fig. 3

M

AACTAGTACGCAAGTTCACGTAAAAAGGGTTTGAAATCGATG

HpaI

EcoRI

ClaI

1

Ptrp

BstEII
Bg/II

amp

BamHI

pKM6   tet

SalI

Fig. 4

EcoRI

ClaI

NdeI

2

Ptrp

HinfI

HinfI

3

amp

BstEII

p6huγ-N1

HinPI

BamHI

SalI

Fig. 5

```
        G  Y  L  R  N  S  R
        GGTTACCTCCGAAACTCGAGCTGAGATCT
```


EcoRI

ClaI

Ptrp

amp

pKM6-CXho

BstEII

XhoI

ΔBamHI/BglII

SalI

Fig. 6

```
        S  Q  M  L  F  R  G  R  R  A  S
        GAGTCAGATGCTGTTTCGCGGTCGACGTGCATCC-
                 Q  V  P
               -CAGGTACCATGAGATCTGGATCC
```


EcoRI

ClaI

Ptrp

amp

p6huγN1-CKpn

HinfI

SalI

KpnI

BglII

BamHI

SalI

Fig. 7

Fig. 8

Fig. 9

Fig.10

ClaI
2
KpnI
BamHI

p6huγNI
-CKpn

ClaI
1
BglII

pKM6

KpnI DIGESTION

ClaI DIGESTION
BglII DIGESTION

500bp FRAGMENT
ISOLATION

———— TCCCAGGTAC
———— AGGGTC

T4 DNA POLYMERASE
FRAGMENT

———— TCCCAG
———— AGGGTC

BamHI DIGESTION
ca. 4800bp FRAGMENT
ISOLATION

CGATG ———— TGAA
TAC ———— ACTTCTAG

GATCC ———— TCCCAG
G ———— AGGGTC

SYNTHETIC DNA FRAGMENT

ACTCAG ———— GTAA
TGAGTC ———— CATTGC

T4 DNA LIGASE

EcoRI   PvuII
ClaI
2
PvuII
PvuII
1
ΔBglII/BamHI
SalI

ptrp6huIFN
-γcβ
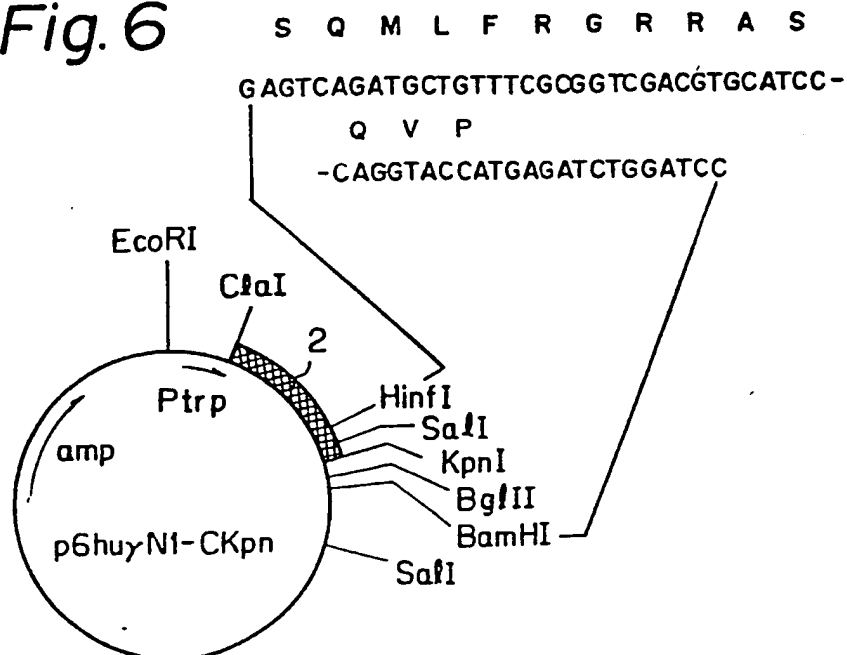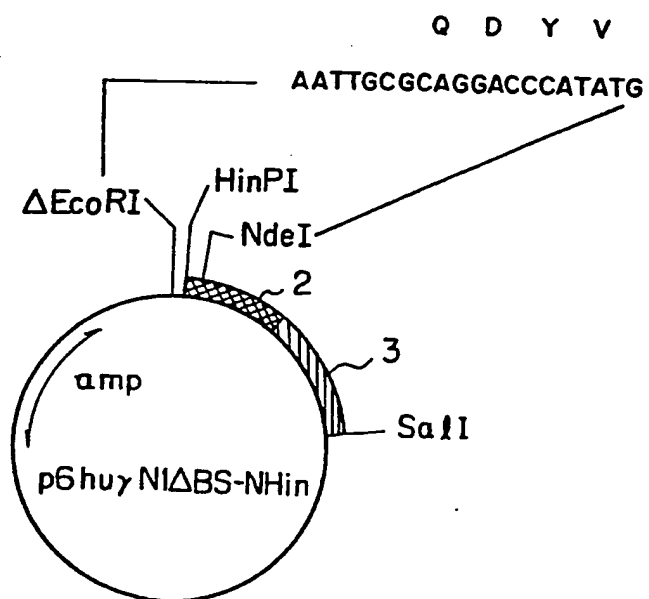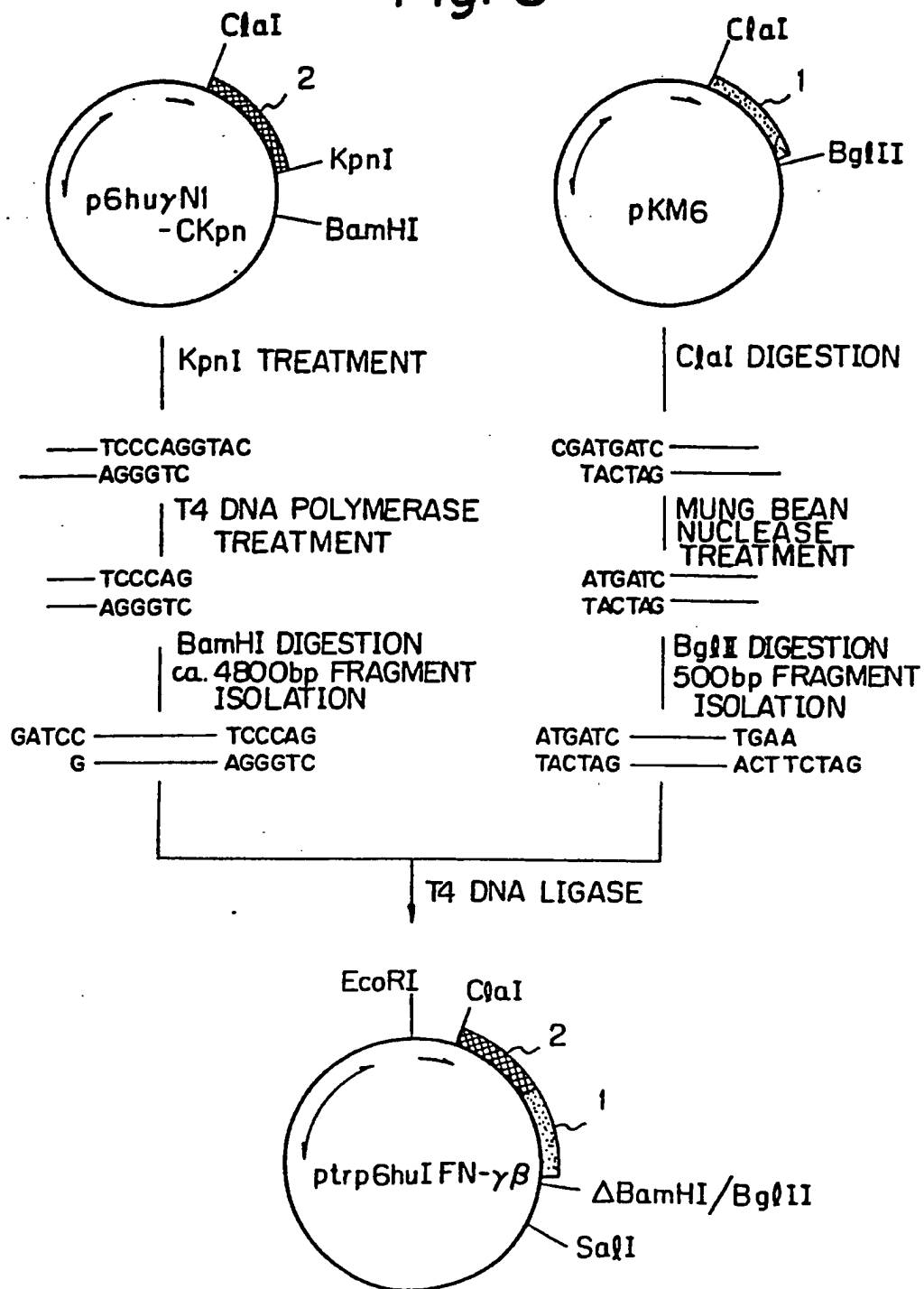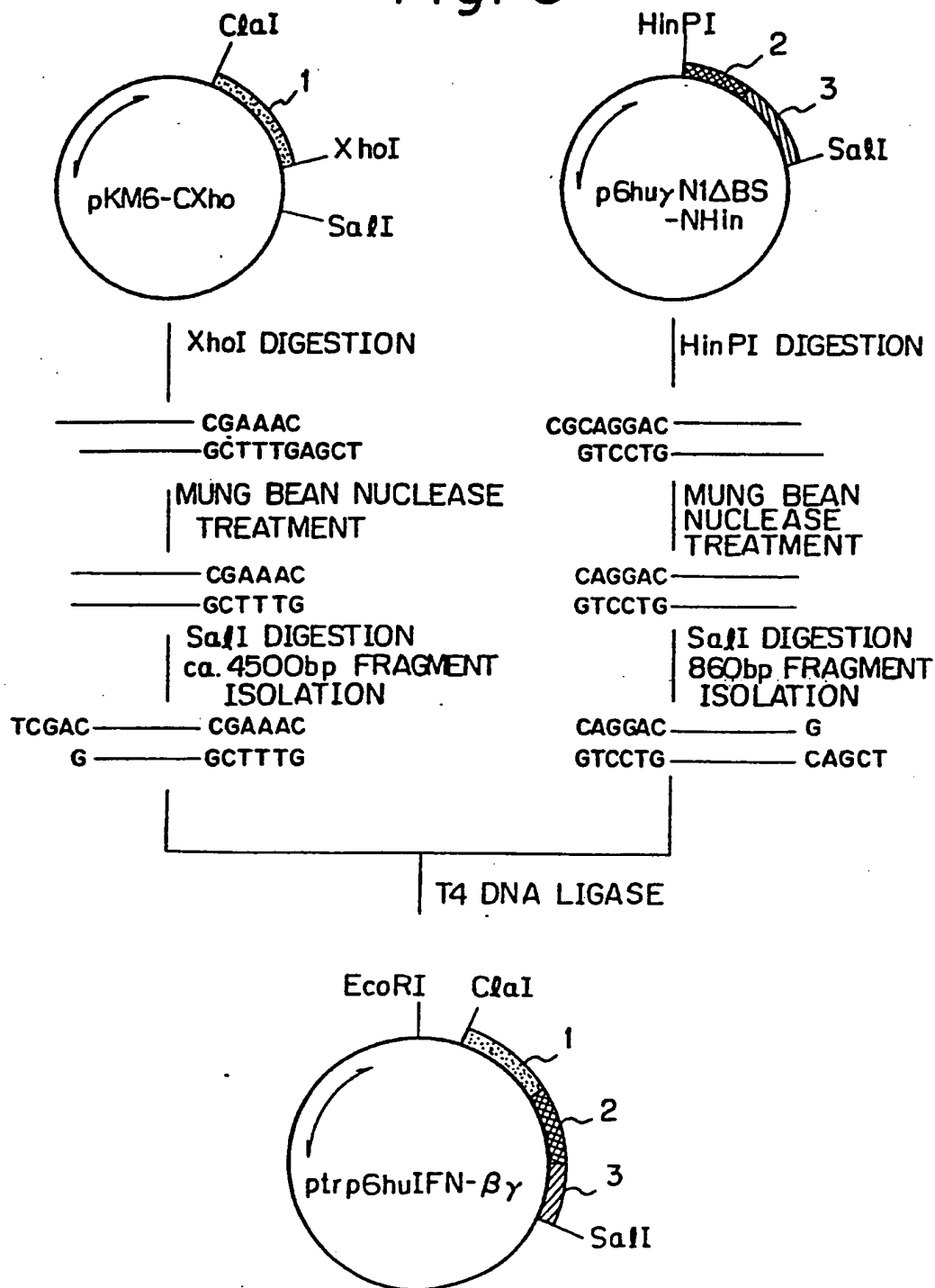
PvuII

Fig. 11

```
  T  Q  L  G  Q  P  K  A  A  K  S  V  T

ACTCAGCTGGGCCAGCCGAAAGCTGCTAAGTCGGTAA

TGAGTCGACCCGGTCGGCTTTCGAGCATTCAGCCATTGC

     PvuII
```
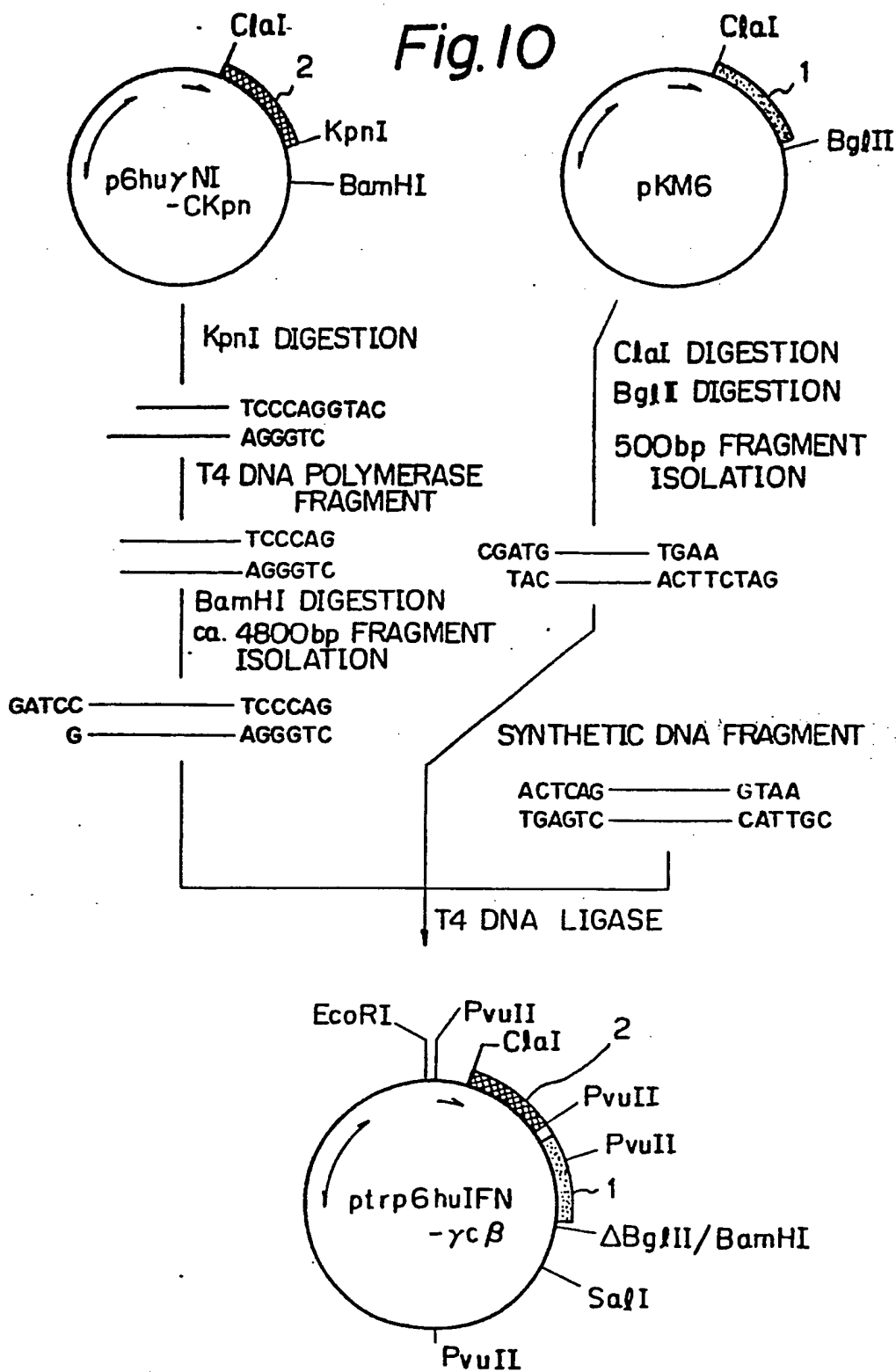
Fig.12

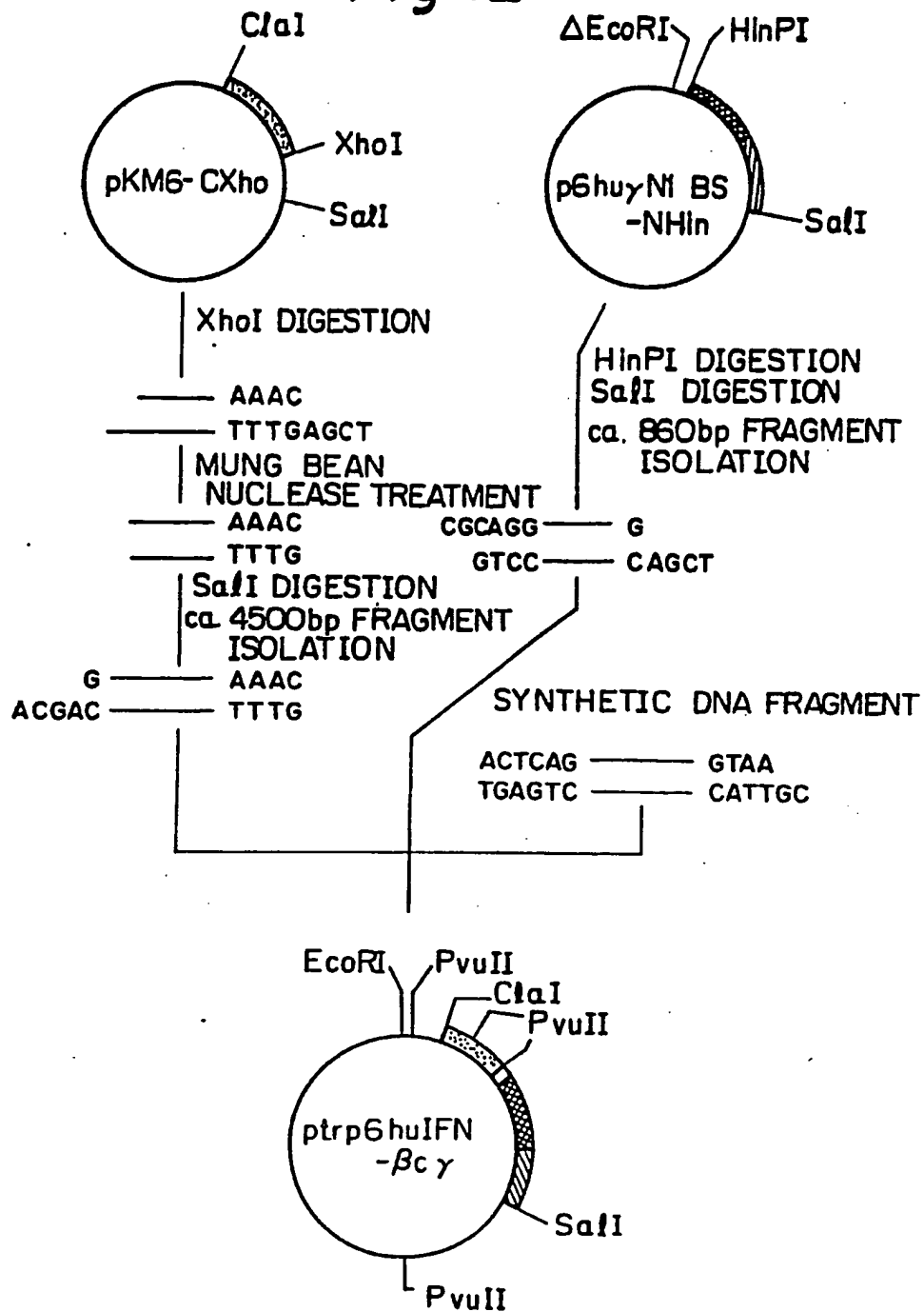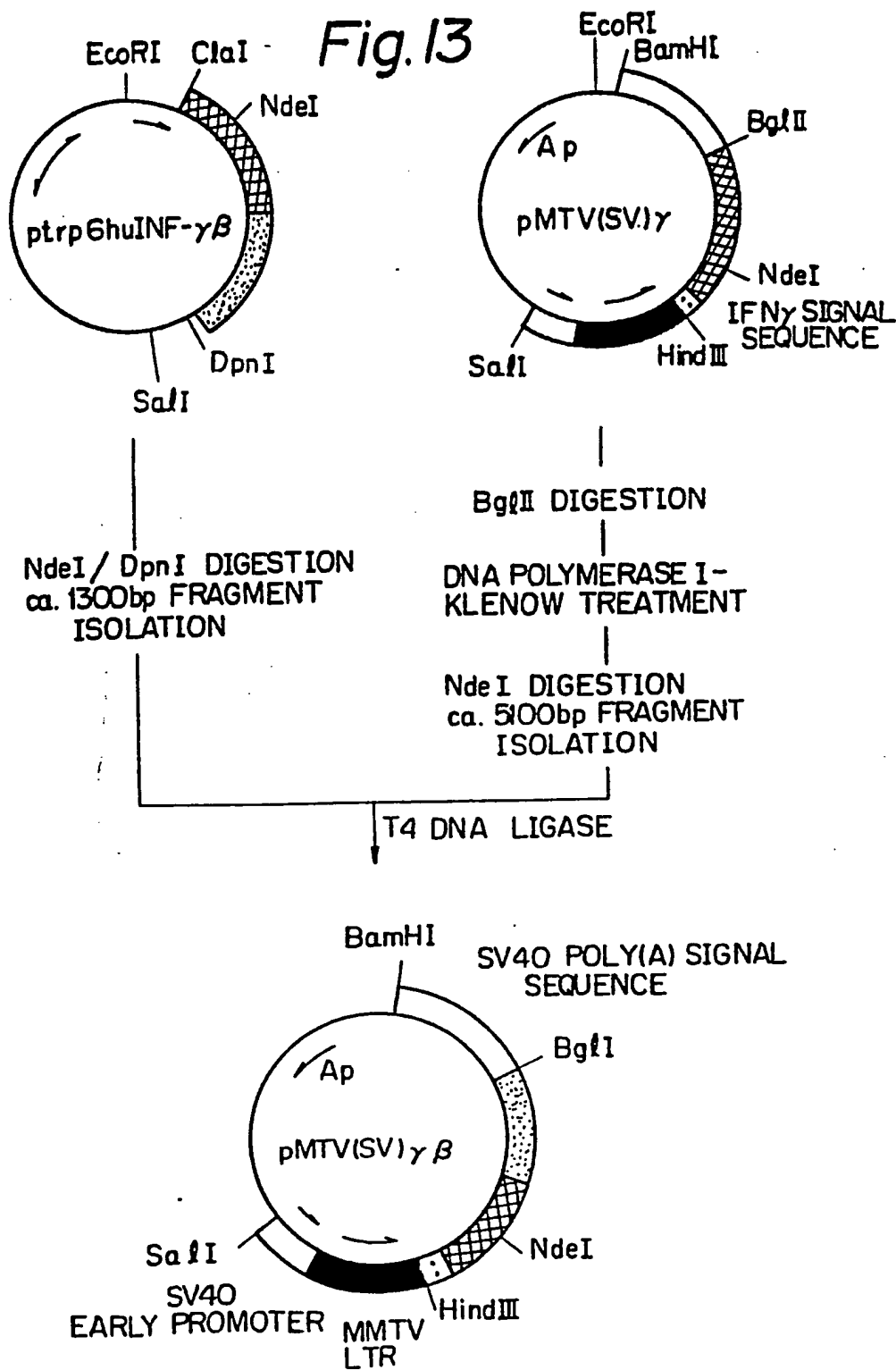## Fig. 13

Fig. 14